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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The principal focus of this project is to characterize the effects of shock and shock-related cell injuries on: 1) cell structure and function; 2) regulation of cell and organelle ion regulation, especially that of $[Ca^{2+}]_i$; 3) the role of $[Ca^{2+}]_i$ in the orchestration of transmembrane signalling in shock and cell injury; 4) the role of cytoskeletal membrane interactions in the changes that result; and 5) the effects of ion deregulation following sublethal injury on altered gene expression. Our strategy is to use both anoxia/ischemia animal and human *in vitro* model systems and an *in vivo* hemorrhagic shock model developed in our laboratories. With this approach, we hope to be able to extrapolate animal data to man. In addition to the use of routine established morphological and biochemical techniques, we will continue to use newly developed technologies such as digital imaging fluorescence microscopy to measure $[Ca^{2+}]_i$ and $[H^+]_i$ in living cells and molecular biology techniques to study gene expression.

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INTRODUCTION

Cellular and molecular changes constitute the fundamental basis of the altered pathophysiology of shock and trauma (1). Such changes range from sublethal to lethal alterations, the inflammatory process, repair and healing, and the concomitant modulation of cell function and secretion with the elaboration of numerous mediators such as cytokines which in turn modulate cell structure and function, further altering cell physiology (2). It is, therefore, of the utmost importance to understand the nature and mechanism of cellular changes which constitute the basis of multiple organ failure. Knowledge of the mechanisms involved could lead to improved diagnosis, prevention and treatment. As we have discussed elsewhere (1), research on shock and trauma must proceed at the cellular and molecular levels. This is not only because the technology for the culture of both animal and human cells has been established, but because cellular and molecular changes occur and can be detected at this level.

Following injury, all cells undergo a characteristic series of responses which initially are sublethal or prelethal (2,3). In some cases, the cell may adapt at this stage resulting in a sublethally altered cell with an altered homeostatic level. In some cases, such prelethally injured cells reenter the mitotic cycle and divide, repairing deficits in the organ, e.g., liver or kidney tubule, and ultimately redifferentiate into normally functioning differentiated G0 cells. With many injuries, however, if the injury state is continued beyond a certain point ("point-of-no-return"), the cell is said to **die** and undergoes a series of degradative reactions collectively known as **necrosis** (2). These stages have been extensively characterized both structurally and functionally in our laboratory and defined as a series of stages of cell injury (1-3).

Briefly, the sublethal or prelethal changes include changes in cell surface conformation, including the formation of numerous blebs, changes in organelle volume including dilatation of the endoplasmic reticulum (ER), and condensation of the mitochondria

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followed later by mitochondrial swelling, clumping of nuclear chromatin, changes in the density and volume of the cytosol, alteration in the arrangement and polymerization of the cytoskeleton (especially involving actin and tubulin), and changes in the phagolysosomal system, notably the occurrence of autophagocytosis. At this stage, the functional alterations typically include diminished or absent ATP synthesis with increased ADP/AMP, leak of such nucleotides to the extracellular space, changes in ion content including increased $[Na^{2+}]_i$ and $[Cl^-]_i$, decreased $[K^+]_i$, changes in pH, loss of mitochondrial membrane potential, and sometimes escape of cytosolic enzymes to the extracellular space or to the circulating blood through the shedding of blebs which seal-off prior to cell death (4-10). Changes in membrane permeability are characterized by modification of membrane phospholipids which seems to account for both cell and mitochondrial swelling. Nuclear chromatin clumping is striking, is probably associated with altered transcription, and may relate to the ongoing changes in cytoplasmic and nuclear pH (3). Reduction of cellular pH is protective for many types of cell injury including anoxia/ischemia (11).

After the cell dies, another series of characteristic/morphologic changes occurs, including breaks in plasma membrane continuity and further mitochondrial swelling with the formation of one or two types of intramatrical inclusions. The first type, flocculent densities, consists of denatured mitochondrial matrix protein and are common to virtually all types of irreversible cell injury. These appear as dense deposits with a fluffy irregular contour and are removed by digestion with proteolytic enzymes. The second type, occurring in some though not all cell injuries (12), consists of the formation of first amorphous and then crystalline calcium phosphate deposits, later converting to calcium hydroxyapatite. These begin as punctate, amorphous densities near the cristae and, in some cases, later grow to eventually fill entire mitochondria with crystalline hydroxy-apatite. This results in calcification which is visible as basophilic areas in hematoxylin-eosin stained sections, or eventually as chalky white deposits visible to the naked eye. This so-called "dystrophic calcification" has long been associated by pathologists with cell injury and cell death in the brain, heart, kidney, pancreas, liver, and other organs. Such extensive calcification can have severe consequences at the organ level, e.g., the calcification of the aortic valve in aortic stenosis and calcification in the kidney forming nephrocalcinosis and calciuria. This association between calcification and cell death is also reflected at the cellular level. Some years ago, we characterized a number of injuries in Ehrlich ascites tumor cells (EATC) at the cellular and subcellular level, consistently noting a good correlation between the amount of Ca^{2+} and cell death (13). At the same time, it appeared to us that such calcification represented

a consequence rather than a cause of cell death, a marker so to speak, but wondered whether or not earlier changes in calcium, especially cytosolic ionized calcium ($[Ca^{2+}]_i$) might, in fact, be involved in the pathogenesis (12-14).

Until recently, it has not been possible to measure $[Ca^{2+}]_i$ in living cells. Two recent major developments have made this possible: the first was the development of video intensification microscopy fostered by the work of Allen (15) and Inoue (16) through which extremely low photon signals, often invisible to the unaided eye, can be imaged using intensified video cameras; the second was the development of fluorescent probes, typically lipid soluble esters, which enter cells and are hydrolyzed by cytosolic esterases and act as specific chelators for ions such as Ca^{2+} (17). When excited at appropriate wavelengths, the photon signal is proportional to the concentration of the chelate. Several such probes have been developed by Roger Tsien and his laboratory for $[Ca^{2+}]_i$. Currently, the most widely used probe is the ester, Fura-2 AM, and several methods have been developed for its use (18,19).

We had earlier proposed a general working hypothesis for the effects of prelethal and lethal cell injury involving a primary role for altered cell ion regulation, especially $[Ca^{2+}]_i$ (5). The development of these new technologies have now made it possible to test this hypothesis in a variety of cells. Meanwhile, the technology of culturing both animal and human epithelia was being developed and, in our laboratory, we established methods for culturing virtually any type of human epithelium important as targets in shock including lung, kidney, liver, pancreas, stomach, small intestine, and large intestine (20). These developments permit not only the application of such technologies to studies of cell and molecular biology following injury, but also the comparison of results in animal models with those observed in humans.

With this background in mind, we established several objectives to characterize the effects of shock and shock-related cell injuries on: (1) cell structure and function; (2) regulation of cell and organelle ion regulation especially that of $[Ca^{2+}]_i$; (3) the role of $[Ca^{2+}]_i$ in the orchestration of transmembrane signalling in shock and cell injury; (4) the role of cytoskeletal membrane interactions in the changes that result; and (5) the effects of ion deregulation following sublethal injury on altered gene expression.

RESULTS

A. Calcium ionophores

In order to study the effects of deliberate introduction of Ca^{2+} into the cytosol, experiments were conducted using Ca

ionophores, including ionomycin and A23187, on human and rat proximal tubular epithelial cells (PTE) *in vitro* . Ca ionophores exert a lethal effect which is clearly dependent on extracellular calcium ($[Ca^{2+}]_e$). When added in the presence of normal $[Ca^{2+}]_e$ (~ 1 mM), the killing is rapid; if $[Ca^{2+}]_e$ is minimized with or without the addition of chelators such as EGTA, there is a marked retardation in the killing curve. The increase of $[Ca^{2+}]_i$ begins essentially immediately and, by 1 min, clear increases in the perinuclear region are seen. These increases progress until, by 10-12 min, marked increases are even seen in the cell nucleus. Cytoplasmic blebbing is apparent and the blebs often contain extremely high concentrations of $[Ca^{2+}]_i$, approximating 1-2 uM, the saturation point of Fura-2 (21). Actually, such concentrations could be even much higher but cannot be accurately measured using current techniques.

These changes are followed by increased expression of c-fos mRNA which increases from a low constitutive level to markedly detectable levels at 10, 20, 30, and 60 min, reaching a peak at about 30 min. In similar fashion, expression of c-myc occurs, reaching a peak approximately at 120 to 180 min. Electron microscopically, cells treated with A23187 show early mitochondrial condensation, cytoplasmic blebbing, nuclear chromatin clumping, increased autophagocytosis, and, soon thereafter, mitochondrial swelling with flocculent densities.

B. $HgCl_2$ nephrotoxicity

$HgCl_2$ is a classic nephrotoxin in animals and humans both *in vivo* and *in vitro*. When administered to a rat at a dose of 4 mg/kg, $HgCl_2$ results in acute reversible renal failure. This is characterized by necrosis in the P3 segment of the proximal tubule and usually accompanied by extensive calcification predominantly within the mitochondria in the form of hydroxyapatite as discussed above (12). After a period of diuresis and beginning recovery, the sublethally injured cells at the border of the necrotic zone enter the mitotic cycle, cover the basal lamina and, during the course of 2 wk, regenerate and become intact PTE.

Among the questions that need to be answered are the following: (1) is calcification only an indicator of cell injury and cell death or does change in $[Ca^{2+}]_i$ precede cell injury; (2) what is the stimulus for regeneration of the sub-lethally injured cells at the margin; and (3) does $HgCl_2$ act as a generic sulfhydryl inhibitor or are there other factors involved? We, therefore, investigated the effects of $HgCl_2$ at 10-100 μM in rat, rabbit, and human PTE. Thus far, most of our studies have been on rabbit PTE, although we have found that similar changes occur in the rat. Addition of $HgCl_2$ gives a dose response curve for cell killing; most of the studies were done with 50 or 100 μM. Using Fura-2, it

was evident that increased $[Ca^{2+}]_i$ occurs within seconds following the addition (21,22). The initial change is diffuse, sometimes perinuclear, and, as time progresses, nuclear accumulation is significant. Interestingly, there is an initial period of increased $[Ca^{2+}]_i$ followed by a brief period of buffering and a later increase. If $[Ca^{2+}]_e$ is reduced, the increase, although present, is markedly buffered. We conclude that most of the increased killing is due to influx from the extracellular space. Using digital imaging fluorescence microscopy (DIFM) and phase microscopy, it was apparent that in many cases the large cytoplasmic blebs that formed early, long prior to cell death, contained extremely high concentrations of $[Ca^{2+}]_i$ saturating the Fura-2.

As blebs could also be produced by modifiers of cytoskeletal elements, such as cytochalasin and vinblastine, we also investigated the patterns of actin distribution using antibodies and phalloidin (23). Following $HgCl_2$ treatment, there were marked disruptions of the normal actin pattern followed by a great diminution of staining intensity. Typically, there was a band of actin at the base of a bleb corresponding, we presume, to the prominent bundles of thin filaments seen in transmission electron microscopy (TEM) (21).

In order to compare these results with those of other sulfhydryl reagents, we investigated the effects of PCMB, PCMBS, and N-ethylmaleimide (21,22). Interestingly, these other sulfhydryl compounds had an entirely different effect in the sense that although $[Ca^{2+}]_i$ increased, the increase seemed to be entirely an effect of redistribution.

C. Models of anoxia/ischemia

As models of anoxia/ischemia, we have utilized uncouplers of mitochondrial oxidative phosphorylation, such as FCCP, inhibition of respiration with KCN, and inhibition of glycolysis with iodoacetate. To simulate total ischemia, we have combined KCN and iodoacetate. Although uncouplers, such as FCCP, resulted in a transient increase of $[Ca^{2+}]_i$ from the normal 100 nM to ~ 200-300 nM, application of KCN or iodoacetate alone resulted in no change. However, addition of both at the same time, inhibiting both respiration and glycolysis, resulted in $[Ca^{2+}]_i$ increases which were slower and less than those seen with membrane damage as in $HgCl_2$, but peaking at ~ 10-15 min in the perinuclear region.

D. Effects of xanthine/xanthine oxidase

Oxidant stress has become an important focus of studies on acute toxic injury as many chemical compounds in the environment, as well as moieties released following shock and trauma, such as the hydroperoxides resulting from membrane lipid

oxidation, result in the formation of active oxygen species. Furthermore, the inflammatory reaction to injury with influx of leukocytes and later macrophages itself produces abundant sources of oxygen radicals. As a model of oxidant stress, we have utilized xanthine/xanthine oxidase (24,25).

These studies showed a dose and time response of change in $[Ca^{2+}]_i$ at 5,10, and 25 mU/ml of xanthine oxidase. The $[Ca^{2+}]_i$ increased in a fashion similar to that described above, often initially in the perinuclear cytoplasm and later in the overall cytoplasm. A response was seen as early as 2 min and reached a maximum, saturating the Fura-2, at 1-2 μm by 60 min. Under the conditions studied, the response of $[Ca^{2+}]_i$ was dependent on $[Ca^{2+}]_e$ in the sense that reduction of $[Ca^{2+}]_e$ with or without addition of EGTA minimized or totally prevented the increase. The lethal response to xanthine/xanthine oxidase was also diminished by either superoxide dismutase (SOD) or catalase addition or almost totally eliminated in the presence of both enzymes.

In order to explore the effects of oxidant stress on gene expression, c-fos was demonstrated by Northern analysis and showed a similar dose response curve, with a peak of c-fos activity between 30 and 60 min (24). The response was greatly diminished by the addition of SOD and also was greatly diminished if $[Ca^{2+}]_e$ was reduced or if EGTA was added. A similar diminution of constitutive c-fos activity was also noted by reduction of $[Ca^{2+}]_e$.

DISCUSSION

A. General

The current experiments have clearly revealed major ion deregulation, focusing on $[Ca^{2+}]_i$, and following immediately upon the imposition of acute models of cell injury related to shock and trauma. We visualize alterations in $[Ca^{2+}]_i$ as a major orchestrator of a number of prelethal and lethal events that occur following injury *in vivo* and *in vitro*, including cytoplasmic blebbing, alterations in the cytoskeleton, ER and mitochondrial swelling, autophagocytosis, changes in organelle volume and membrane permeability including mitochondria and ER. Furthermore, they include altered genomic function in the prelethal phase including increased transcription of genes related to cell division/ differentiation such as c-fos. These changes undoubtedly also relate to the liberation of cytokines and mediators of the inflammatory process. It is also likely that these genomic changes relate to cell division, regeneration, and differentiation, implicit in the process of repair and recovery. These studies have been made possible by the introduction of *in vitro* methods for the culture of important human target epithelia in both animals and humans for extrapolation to studies of human shock and trauma using

immediate autopsies or animal models *in vivo*. The use of fluorescent probes and DIFM represents another major advance with additional progress in this area being made very rapidly. Using these technologies, a variety of subcellular and molecular changes can be visualized as a function of time in living injured cell models, correlated with biochemical and functional stages, and ultimately with studies in patients or animal models *in vivo*.

B. Mechanisms of $[Ca^{2+}]_i$ deregulation

Normally $[Ca^{2+}]_i$ is maintained in many cells, in this case rat PTE, at levels approximating 100 nM. This regulation is a function of a variety of transport and binding systems which regulate the cytosol at this low concentration in the face of ~ 1 uM $[Ca^{2+}]_e$. These include the plasma membrane, the ER or specialized subsets thereof, the mitochondria, and possibly the nucleus. In addition, a variety of calcium-binding proteins, notably calmodulin, also regulate $[Ca^{2+}]_i$ and mediate many of the important physiologic and pathologic events. At the plasma membrane, the control of $[Ca^{2+}]_i$ is two-fold. The first is specific and nonspecific Ca^{2+} entry through chemically- or electrically-gated channels or through pathologic modification of Ca^{2+} entry, e.g., modification of membrane/protein sulfhydryls, modification of membrane phospholipid, or insertion of channels such as the C5-9 complex of complement. Additionally, exogenous or endogenous Ca ionophores can readily mediate transfer of Ca^{2+} across the cell membrane if $[Ca^{2+}]_e$ levels are normal. Balancing these are Ca^{2+} extrusion systems, including Ca-ATPases, which vary between different cells, and Na^+/Ca^{2+} exchange, whereby an increase or decrease of $[Na^+]_i$ results in subsequent rapid alterations in $[Ca^{2+}]_i$. Na^+/Ca^{2+} exchange can also be readily induced by inhibition of the Na/K-ATPase with cardiac glycosides, such as ouabain, or depletion of ATP, thereby implying that Na/K-ATPase itself controls $[Ca^{2+}]_i$ indirectly. This is more well-developed in some cells than in others; however, we have shown that, in the PTE, it has a significant role. Finally, because of the phenomenon of Na^+/H^+ exchange, which is often amiloride-sensitive, changes in $[H^+]_i$ can affect $[Na^+]_i$ or vice versa. This may play a major role in the protection of a variety of injuries by acidosis and may also be implicated in the demonstrated protection of cells by reducing the pH of perfusates during reflow. The mitochondria and ER also possess high and low affinity sites for the rapid uptake and/or release of Ca^{2+}. In the case of the mitochondria, the affinity is lower, but the capacity is much higher because of the relative membrane area of the mitochondria in many cells, including the proximal tubule. In the presence of phosphate, excessive amounts of transported Ca^{2+} can precipitate in the mitochondrial matrix in the form of calcium phosphate and convert to hydroxyapatite. This represents an extensive buffer system whereby the mitochondria can accumulate vast amounts of Ca^{2+}, raising total tissue levels to

grossly visible Ca^{2+} deposits. Activation of this system is seldom if ever seen in prelethally altered cells; however, following lethal injury with agents that do not specifically interfere with mitochondrial uptake, such as PCMBS or complement, mitochondrial calcification can be extensive with virtually every mitochondrion filled with calcium phosphate deposits. Similar calcium phosphate deposits can be seen in renal tubules treated with ouabain to inhibit the Na/K-ATPase. Such deposits are not, however, seen in injuries such as anoxia or chemical analogs thereof, as mitochondrial uptake is inhibited; however, following reflow into sublethally altered areas, the subsequent necrosis is often accompanied by calcification. The ER possesses higher affinity sites best characterized in contractile cells. In epithelia, such as the PTE, a common early response to injury is dilatation of the ER and ribosomal dissociation. The mechanism of this has been incompletely established. One hypothesis is that the dilatation is a result of uptake of influxed Ca^{2+} with subsequent distention of the ER and calcium protease interactions, resulting in ribosomal dissociation. This latter phenomenon needs much further study. Finally, calcium-binding proteins, such as calcium-calmodulin complexes, may mediate not only buffering of $[Ca^{2+}]_i$, but the effect may result in modification of microtubules and filaments or activation of phospholipase. The use of DIFM has, for the first time, strongly suggested that the nucleus can be an important site of increased $[Ca^{2+}]_i$. This increase is normally occurring at a time when alterations in the conformation of nuclear chromatin and nuclear structure are occurring and precedes the activation of genes, such as c-fos and heat shock proteins. This is further discussed below, but may relate to calcium-activated nuclear endonucleases and DNA strand breaks.

C. Bleb formation and cytoskeleton

The formation of cytoplasmic blebs is a common accompaniment of acute cell injury both *in vivo* and *in vitro*. *In vivo*, such cytoplasmic blebs are most conspicuous in epithelia that line cavities or are adjacent to lumens, such as PTE or hepatic parenchymal cells. We have known for some time that these blebs form early, long before cell death, pinch off and detach to the medium with membrane sealing, and that they are compatible with cell survival, the loss of membrane later being repaired to restore the normal conformation, e.g., the microvillus brush border in the PTE. These blebs may have physiologic significance *in vivo*. In the kidney, they are discharged to the lumen and form casts in the more distal parts of the nephron which have been postulated to produce tubular obstruction, increased tubular pressure, and decreased glomerular filtration rate, contributing to acute renal failure. In the liver or other cells, the detachment of such blebs and their subsequent lysis in the plasma or extracellular space results in the release of cytosolic enzymes such as LDH or serum

glutamic pyruvate transferase, indicating injury without the necessity of necrosis in the contributing epithelium. Because of their prominence and physiologic significance, we have spent considerable effort in characterizing these blebs (21). The blebs form rapidly, are of low viscosity and are typically free of organelles. As a bleb increases in size, in most cases, the neck narrows sequentially, finally sealing off with release of the bleb, thus representing a type of stimulated apocrine secretion, whereby part of the cytoplasm is liberated to the extracellular space. It was most interesting to note in our imaging studies of $[Ca^{2+}]_i$ that these blebs are calcium-related, e.g., they form rapidly after Ca ionophores such as ionomycin and that their size and number, with other sorts of injury, is proportional to the extent and duration of the increase in $[Ca^{2+}]_i$. From our studies, it appears that bleb formation begins with small and infrequent blebs at $[Ca^{2+}]_i$ concentrations of 300-400 nM; however, when a concentration of 1 μM is approached, the blebs are extremely large and numerous.

Since previous studies in our laboratory had shown that modifiers of cytoskeleton, such as cytochalasin and vinblastine, could induce similar if not identical blebs, it seemed reasonable to hypothesize that cytoskeletal alterations and cytoskeletal membrane interactions might be the mechanism following other types of shock-related injury. We, therefore, investigated the patterns of actin and tubulin during the process of bleb formation and found marked alterations, using either monoclonal antibodies to actin or actin phalloidin fluorescent markers (23). Actin is absent in the region of the bleb except for a thin peripheral rim around the surface; however, a band of actin filaments does occur at the base. This event appeared to correlate with the exclusion of organelles from the bleb itself and we hypothesize that the contractile band of actin filaments is involved in the final pinching off in the detachment process. Furthermore, after certain injuries, there is diffuse irregularity in the actin cables throughout the cytosol and diminution in staining. As it has been known that calcium-mediated proteases can modify elements of the cytoskeleton, we investigated effects of leupeptin and antipain and found that pre- or concomitant treatment with these calpain inhibitors resulted in amelioration of bleb formation and the cytoskeletal alterations (26). Furthermore, some reduction of loss of viability occurred, suggesting a role of the cytoskeleton and possibly blebs in the ultimate cell death.

D. Autophagocytosis

Autophagocytosis is best known and characterized as a process whereby organelles such as mitochondria, ER, and peroxisomes are turned over as part of normal cell function. With many types of cell injury, autophagy increases. It consists of the budding of parts of the cytoplasm into the lumens of the ER,

followed by pinching off, subsequent fusion with primary and/or secondary lysosomes with digestion. In other words, autophagy is analogous to blebbing at the cell surface except that the microblebs occur and pinch off into the cavity of the ER. We had known previously that modification of the cytoskeleton could increase autophagocytosis and subsequently found that Ca ionophores could also do the same in a similar fashion, as well as increase proteolysis. Because the increased $[Ca^{2+}]_i$ precedes autophagy, it is reasonable to assume that this process is also mediated through Ca^{2+} or calcium-calmodulin effects on the cytoskeleton at the organelle level. Increased autophagy and proteolysis are extremely important in the severe catabolic states that occur *in shock* and have been demonstrated *in vivo* in liver, kidney, and skeletal muscle.

E. Organelle changes

As mentioned above, volume changes, including condensation and later swelling, occur in the mitochondria and swelling in the ER. These volume changes are inferred to result from calcium-mediated attack on mitochondrial inner membranes through protease and/or phospholipase. We had previously demonstrated *modification of membrane* phospholipids, including cardiolipin in mitochondria, and increased permeability to ions within the cytosol which can also be demonstrated *in vivo* by performing swelling experiments.

F. Genomic changes

A new facet of this study is the probable mediation of genomic changes by $[Ca^{2+}]_i$ and/or changes in $[H^+]_i$ (27-29). One phase of cell injury, long prior to cell death, is alterations in the conformation of chromatin clumping and changes in nucleolar aggregation. These are preceded by changes in $[Ca^{2+}]_i$ and sometimes by changes in pH. Genomic changes are essential in order to produce regeneration of cells near necrotic areas which must subsequently reenter the mitotic cycle, divide, and ultimately differentiate back to normal structure and function. As a beginning model of genomic changes related to cell division/differentiation, we chose to investigate the expression of c-fos following oxidant stress (24). Our results showed a time- and dose response curve of c-fos expression following xanthine/xanthine oxidase treatment with a peak of approximately 30-35 min with return to normal levels by ~ 1-2 hr. This expression of c-fos was simulated by Ca ionophore treatment. Since it is preceded by increased $[Ca^{2+}]_i$, we hypothesize that $[Ca^{2+}]_i$, in some way, initiates the process. This is made even more interesting by the localization of $[Ca^{2+}]_i$ in the nucleus following several of the injurious agents. Cerutti and his group found that pretreatment with Quin-2 to buffer Ca^{2+} ameliorated the expression

of c-fos and the production of DNA strand breaks (30,31). One reasonable hypothesis is that Ca-activated nucleases produce DNA strand breaks which, in turn, incite poly-ADP-ribosylation of fos protein. Fos protein, probably together with jun products including P39, can form heterodimers which act as inhibitors of the trans-mediated promoter regions of the c-fos gene. In other words, poly-ADP-ribosylation of fos protein can remove a negative feedback on fos expression and result in increased fos production. The function of the fos gene in normal and abnormal physiology is under considerable study in many laboratories. We infer that, in the case of acute cell injury, these changes may relate to normal repair and regenerative activity.

CONCLUSIONS

Although much more work is needed, data from the experimentation we have performed and described above have led us to make the following conclusions.

1. Shock and shock-related cell injuries consist of lethal and sublethal events that relate to changes in the ion regulation of cells, including $[Ca^{2+}]_i$. We infer that changes in $[Ca^{2+}]_i$ orchestrate a variety of cytoplasmic and genomic changes important in sublethal and lethal cell injury as well as in cell repair.

2. Increased $[Ca^{2+}]_i$ may result primarily from: a) increased influx of Ca^{2+} at the plasma membrane, e.g., Ca ionophores, complement; b) decreased efflux of Ca^{2+} at the plasma membrane, e.g., inhibition of Na^+/Ca^{2+} exchange or the Ca-ATPase; c) redistribution of Ca^{2+} from organelles, e.g., mitochondria and ER, seen with sulfhydryl reactive compounds, such as N-ethylmaleimide and PCMBS and Ca ionophores in Ca^{2+}-free media; d) both (a) and (b), e.g., $HgCl_2$, anoxia, KCN + IAA, and oxidant stress.

3. Protection by reduction of $[Ca^{2+}]_e$ is greatest in (2a) but also occurs in (2c).

4. Increased $[Ca^{2+}]_i$ precedes bleb formation with a threshold of approximately 300 nM.

5. Increased $[Ca^{2+}]_i$ precedes the induction of altered gene expression including c-fos.

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